Peripheral Prostanoid Levels and NSAID Analgesia:

Replicate Clinical Trials in a Tissue Injury Model

for Pharmacodynamic Studies Section

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Abstract

Background: NSAID analgesia is generally attributed to peripheral suppression of cyclooxygenase (COX) enzymes leading to decreased products of the arachidonic acid cascade. This study evaluated the *in vivo* relationship between levels of prostanoids at the site of tissue injury and analgesia following systemic or local NSAID administration in a clinical model of tissue injury.

Methods: Subjects in two replicate clinical trials had one or two mandibular third molars removed, a microdialysis probe implanted at the surgical site for measurement of i.r. PGE_2 or i.r. TxB_2 and pain measured concurrently. In the first study, ketorolac was administered at pain onset in a 30 mg dose IM, 1 mg IM or 1 mg submucosally at the extraction site in comparison to placebo; in the second study subjects received either ketorolac 30 mg IV or placebo at pain onset.

Results: PGE_2 was detectable in the first postoperative sample, decreased over the next hour, and then increased significantly coincident with the onset of postoperative pain. Administration of 30 mg ketorolac produced parallel decreases in pain, PGE_2 levels, and TxB_2 at the surgical site. Administration of 1 mg ketorolac IM or directly at the surgical site was analgesic but without measureable effects on PGE_2 levels.

Conclusion: The temporal profile of PGE_2 and TxB_2 in the immediate postoperative period is consistent with constitutive COX-1 initially followed by an increase in PGE_2 due to expression of COX-2. The temporal association between NSAID analgesia and decreased prostanoids at the site of injury is consistent with a dual COX-1/COX-2 peripheral site of action. The analgesic effects of 1mg ketorolac without a reduction in PGE_2 at the site of injury suggests an additional central site for NSAID analgesia

INTRODUCTION

Acute pain and the other inflammatory processes in response to tissue injury are modulated by locally-released mediators acting synergistically to produce plasma extravasation and sensitize peripheral nociceptors, resulting in hyperalgesia. The essential role of prostaglandins (PG) derived from cyclooxygenase (COX) during acute inflammation is supported by elevated PG levels and hyperalgesia in carrageenan-inflamed rat paws, both rapidly reversed by dosing with the NSAID ketorolac, the selective COX-2 inhibitor celecoxib, and also within 2-3 hours after administration of an anti-PGE_2 antibody.[1] These data suggest that maintenance of a hyperalgesic state after tissue injury requires continuous production of prostanoids by COX-2. The complex biochemical interactions of short-lived inflammatory mediators following tissue injury, combined with the neural release of substance P and the process of plasma extravasation results in a positive feedback loop continually refueling the inflammatory process.[2] The continued synthesis or release of these mediators contributes to the prolonged time course of inflammation, which far exceeds the initial stimulation.

While eicosanoids are widely distributed and are formed by virtually every mammalian tissue that has been examined, differentiation results in each cell type producing its own characteristic pattern of metabolites depending on the complement of enzymes present and their relative abundance.[3] Similarly, the biologic activity of specific eicosanoids vary among cell types, as well as from organ to organ, acting as both intracellular second messengers and intercellular local mediators proximate to their site of synthesis before dilution in the circulation. Tissue injury releases arachidonic acid from phospholipid stores, which are acted upon by COX and other enzymes of the prostanoid pathway to result in the local formation of one or two major products, depending on the enzymes that are constitutively present. Prostaglandin E_2 (PGE_2) is

the predominant eicosanoid released from endothelial cells of small blood vessels,[4] producing vasodilatation[5] and hyperalgesia.[6] Thromboxane A_2 (TxA_2) is the predominant eicosanoid product of platelets,[7] resulting in platelet aggregation[8] and vasoconstriction.[9]

While PGE_2 is produced by the actions of both COX-1 and COX-2, TxA_2 is a product of COX-1 in most tissues, permitting indirect assessment of the relative activities of the two COX isoforms by measuring the differential production of PGE_2 and TxA_2 at or close to their site of formation. Thromboxane B_2 (TxB_2) is the physiologically stable metabolite of TxA_2 and is frequently used as a surrogate marker for TxA_2 levels at the site of tissue injury.[10,11]

Examination of the relationship between tissue injury, inflammatory mediators and clinical pain is facilitated by the adaptation of microdialysis methodology to the oral surgery model. Hargreaves and colleagues demonstrated the presence of bradykinin in perfusate collected following third molar extraction, and its suppression by glucocorticoids[12] and the NSAID flurbiprofen.[13] More recently, they have reported that pretreatment with flurbiprofen suppresses prostaglandin levels in comparison to placebo with a concommitant suppression in pain.[14] Although these studies have demonstrated inhibition of synthesis of inflammatory mediators when administered prior to tissue injury, the dynamic relationship between prostanoid release, the development of acute pain, and the subsequent suppression of inflammatory mediators in relation to NSAID analgesia has not been described.

The objective of these two studies was to characterize the time course of peripheral levels of the prostanoids PGE_2 and TxB_2 and their relationship to clinical pain and NSAID analgesia. PGE_2 levels were interpreted as indicative of both constitutive COX-1 and inducible COX-2 activity, while TxB_2 served as an indicator of COX-1 activity. Administration of the dual COX-1/COX-2 inhibitor ketorolac at the usual therapeutic dose (30 mg) permitted examination of the

peripheral effects of NSAID analgesia on prostanoid levels at a site of tissue injury. Administration of a dose normally considered subtherapeutic (1 mg) at the site of injury was intended to produce high local drug concentrations while avoiding blood levels sufficient to have effects at other sites. A parallel group of subjects received 1 mg ketorolac as a control for the local route of administration. The second study was performed to replicate and extend the findings of the first study to include measurement of TxB_2 and the IV route of administration. The results observed in a sensitive clinical model of tissue injury and acute pain are supportive of both a peripheral and central site of NSAID analgesia.

METHODS

Study population. The study was reviewed and approved by the Institutional Review Board of the National Institute of Dental and Craniofacial Research, National Institutes of Health. Subjects were dental outpatients undergoing the surgical removal of bony impacted mandibular third molars. A complete medical history was elicited and an oral examination performed, including a panoramic radiograph, to confirm the need for third molar removal. The surgical and experimental procedures were explained verbally and in writing, and informed consent was obtained prior to study enrollment. Eligible subjects were ASA physical status Class I, aged 18 to 35 years, with indication for the extraction of at least one partial bony mandibular third molar. Exclusion criteria included a clinically significant medical history, chronic use of medications that would obscure assessment of the inflammatory response (such as antihistamines, NSAIDs, steroids, and antidepressants), pregnant or lactating females, and patients unwilling to undergo the data collection procedures. Additional exclusion criteria were a symptomatic tooth suggesting infection or inflammation and unusual surgical difficulty encountered during the surgical procedure.

Procedures and evaluations. A single surgeon performed the surgical extraction of 1-2 third molars using standard surgical techniques, intravenous sedation with midazolam, and local anesthesia (2% lidocaine with epinephrine 1:100,000). After extraction, a microdialysis probe (CMA/20 Microdialysis Probe, CMA/Microdialysis, North Chelmsford MA) was placed along the buccal aspect of the mandible, beneath the mucogingival flap which was elevated for the surgical procedure. The probe fiber consists of a 10 mm flexible, non-metallic semipermeable dialysis membrane with a molecular cut-off ranging from 3000 to 20,000 daltons.[15] The probe and polyethylene tubing (PE 50) were secured to an adjacent tooth with silk suture and the flap closed in the usual fashion using 3.0 chromic gut suture. Sterile lactated Ringer's solution was pumped at 10 microliters per minute and samples collected at 20-30 min intervals following completion of surgery, but prior to pain onset. At the end of each sample collection interval, patients provided pain ratings using a 100 mm visual analog scale and reported on signs of inferior alveolar nerve anesthesia ("numb," "tingling," or normal), and any adverse effects.

Subjects requesting analgesics following offset of local anesthesia and pain onset were administered ketorolac or placebo randomly with the dose and route of administration based on the study design. Dialysate samples were then collected over the course of the observation period at 15 minute intervals. Samples were collected in an opaque collection vial, placed on dry ice following the collection period, and stored at -70^{o} C until assayed by enzyme immunoassay using commercially available enzyme immunoassay kits for immunoreactive (i.r.) PGE_2 or i.r. TxB_2 (Cayman Chemical Company, Ann Arbor, MI) following the manufacturer's recommended methods.

At the conclusion of the study, the microdialysis probes were removed and patients discharged in the care of a responsible adult with a standard NSAID analgesic for pain control, flurbiprofen 50 mg.

Study design. Two prospective, randomized, controlled, double-blind, parallel groups design studies were performed sequentially. In the first study, a single impacted third molar was removed, a microdialysis probe and drug infusion tubing placed submucosally, and samples collected every 30 min with concurrent reports of pain and anesthesia of the inferior alveolar nerve. At the report of moderate pain, usually consistent with loss of the subjective signs of anesthesia, subjects received one of the four interventions by IM or submucosal administration and remained in the clinic for an additional 180 min for continued sample collection and pain assessment every 15 min. In the second study, both mandibular third molars were removed and microdialysis probes placed bilaterally, samples collected every 20 min with concurrent monitoring for the loss of anesthesia and the onset of postoperative pain, and the interventions administered intravenously at the onset of moderate pain. Microdialysis samples were collected for an additional 180 min at 15 min intervals with concurrent pain measurements.

Study drugs. Drug was randomly assigned and dispensed by the NIH Pharmaceutical Development Service. Syringes or capsules containing matching drug or placebo were prepared under sterile conditions and allocated on the morning of the surgical appointment. In both studies the drug and matching placebos were administered in a double-dummy fashion such that subjects received drug or placebo by both routes of administration. In the first study, the interventions were administered at pain onset: 1 mg of ketorolac administered submucosally into the extraction site, 1 mg of ketorolac administered IM for comparison to the same dose administered systemically, 30 mg ketorolac IM as a positive analgesic control, or placebo at both

sites. A lactated Ringers solution identical in appearance and volume to the drug solution was used as a placebo for submucosal administration. In the second study, the interventions were administered IV at pain onset, either 30 mg ketorolac or placebo. Agents were administered under double-blind conditions.

The injectable NSAID ketorolac tromethamine was used as the experimental agent and as a positive control in both studies. It is a potent inhibitor of the both the COX-1 and COX-2 pathways of arachidonic acid metabolism[16] and is considered primarily a peripherally-acting analgesic.[17] Intramuscular administration of ketorolac results in peak plasma concentrations in humans within 45-50 minutes, with a plasma half-llife of 5-6 hours.[18,19] Ketorolac reaches peak plasma concentration in approximately five minutes after intravenous infusion, with an average plasma half-life of 4.5-5 hours.[20] Ketorolac was administered submucosally in the first study to differentiate direct suppression of COX-1 and COX-2 at the site of tissue injury from possible actions at other sites

Statistical analysis. Previous studies by our group comparing a variety of NSAID analgesics to placebo have demonstrated a 40-50 percent difference in pain report at one hour post-surgery,[21-23] with an average standard deviation of 15 on a 100 mm visual analog scale (VAS). A sample size of 30 per group was calculated for equal group sizes based on an alpha error of 5 percent with 80 percent power, factoring in a 10 percent attrition rate.

Data were stored electronically and analyzed using BMDP software (SPSS, Chicago, IL). Subjects failing to enter the principle observation period following extraction of mandibular molars were excluded from analysis, for example: a rating of unusual surgical difficulty determined by the oral surgeon at the time of surgery, or nerve damage resulting in persistent lip anesthesia. Subjects experiencing adverse effects unrelated to the surgical procedure were

included in the analysis. Subjects receiving rescue medication in the 4 hr postoperative observation period were analyzed by intention-to-treat with carry-over VAS scores at each subsequent time point.

Data were analyzed for the mean and standard deviation at each observation for pain intensity, PGE_2 and TxB_2 levels. Pain scores over time were compared among groups by repeated-measures analysis of variance (ANOVA) with post-hoc tests at individual time points to determine the onset of analgesia in each group. Change in mediator levels between baseline and timed specimens were also evaluated by repeated-measures ANOVA with post-hoc testing of the individual time points**.**

RESULTS

Characteristics of the sample (age, sex, height, weight, anesthetic doses, surgical difficulty and duration, and severity of starting pain) were similar across treatment groups in both studies (Table I). The average time to administration of the study drugs; i.e, pain onset, was similar in both studies: 192.9 $\pm$ 43.3 min for the first study and 129.2 $\pm$ 41.8 min for the second study. Of the 125 subjects enrolled in the first study, 62 sets of usable microdialysis samples were collected. A total of 57 subjects completed the second study, with 33 sets of PGE_2 samples and 26 sets of TxB_2 samples collected. Missing samples were due to probe failure causing any of the following: no perfusate output, blood contamination, or inadequate sample volume to permit assay in duplicate. The results of the subjective pain evaluations are reported for all subjects; only subjects with evaluable microdialysis samples are reported for the prostanoid levels.

Time course of PGE_2 and TXB_2 levels following tissue injury

PGE_2 was detectable in the initial postoperative sample, decreased in the samples collected over the next hour, and then increased significantly over time in the last sample collected

coincident with anesthetic offset and report of moderate to severe pain (Figure 1). A similar pattern was seen in the second study. TxB_2 levels in the second study fluctuated within the limits of the assay variability over the first postoperative hour but did not change following tissue injury (Figure 4C).

Effect of ketorolac on pain and PGE_2 levels at the site of injury

Administration of 30 mg ketorolac IM resulted in a significant analgesic effect by the first observation, 15 min after drug, and reached near maximal levels by 60 min (Figure 2A). The effect over time, as measured by the sum of the pain intensity difference scores, was significantly greater than the other three groups. The analgesic onset for the 1 mg ketorolac IM dose was slower, but resulted in analgesic onset by 30 min (Figure 2A) and significant analgesia over time in comparison to placebo (Figure 2B). Administration of the same dose submucosally directly at the surgical site did not produce detectable analgesia until the 45 min observation (Figure 2A) but also resulted in a significant overall analgesic effect in comparison to placebo (Figure 2B).

PGE_2 levels in the microdialysate, adjusted for the time delay from the probe tip to the collection vial, decreased gradually, reaching significantly lower levels in the 30 mg ketorolac IM group in comparison to placebo by 60 min (Figure 3A). Administration of 1 mg ketorolac, both at the extraction site or IM, did not alter PGE_2 levels in the dialysate from the initial sample at pain onset over the 60 min post-drug administration (Figures 3B, 3C). Subjects were permitted rescue medication after the 60 min observation which lowered PGE_2 levels over the remainder of the observation period (data not shown), confounding any differences between the active drug groups and placebo after that point.

The second study demonstrated very rapid analgesic onset following IV administration of 30 mg ketorolac (Figure 4A) with the overall analgesic effect over the 180 min observation

demonstrated clear separation between this dose of ketorolac and placebo ($P<0.01$). PGE_2 levels continued to increase in the placebo group following placebo administration (Figure 4B) but then decreased over the remainder of the observation period as increasing numbers of subjects requested rescue analgesic (Figure 4B). IV administration of ketorolac resulted in a significant decrease in both PGE_2 and TxB_2 levels for the 15 – 60 min post-drug samples (Figure 4B, 4C) and continued to decrease to the limits of detection over the remainder of the 180 min observation period. Statistical comparison was not made between groups after the 60 minute sample as increasing numbers of patients in the placebo group received rescue analgesic for unrelieved pain which lowered subsequent PGE_2 and TxB_2 levels.

DISCUSSION

These replicate studies were designed to evaluate the relationship between locally released prostanoids, pain, and NSAID analgesia in a well-characterized clinical model which permits simultaneous assessment of acute pain and levels of inflammatory mediators at a site of tissue injury. The temporal profile for PGE_2 demonstrated in the first study is consistent with constitutive COX-1 activity at the site of tissue injury followed by enhanced PGE_2 formation with the expression of COX-2 over the first 2-3 hr following surgery. This profile was replicated in a subsequent study with a similar design evaluating the in vivo selectivity of celecoxib[24] and is consistent with elevated expression of COX-2 mRNA at 60-120 after oral surgery in tissue taken from the extraction site.[25]

Ketorolac suppresses both COX-1 and COX-2, which was demonstrated by a reduction in levels of PGE_2 at the site of injury following the 30 mg dose when administered IM (first study) or IV (second study). These findings are suggestive of a functional relationship between PGE_2 levels at the site of injury and pain, but do not permit differentiation between a peripheral effect

and distribution to the CNS with a possible additional central mechanism of action.[26-30] Administration of a 1 mg dose both at the site of injury and IM resulted in analgesic activity without a detectable effect on peripheral PGE_2 levels. Although the analgesic effect was delayed compared to a 30-fold greater dose, the maximal analgesic effect was comparable (Figure 2). These findings are supportive of a central site of action of ketorolac detectable at low doses and suggest that the peripheral effect observed with the 30 mg dose is due to the higher concentration being distributed widely to act on COX at the site of injury.

Animal studies are supportive of this concept. The potency of ketorolac administered intrathecally to rats is approximately 200-fold greater than when administered systemically (IP).[26] Ketorolac penetrates to the central nervous system when administered orally to mice as early as 30 minutes following administration, reaching levels approximately $1/30^{th}$ of plasma concentrations.[20] Peripheral inflammation results in COX-2 expression in the dorsal horn of the spinal cord, elevating PGE_2 levels in the cerebrospinal fluid.[30] Spinal administration of a COX-2 inhibitor decreases peripheral inflammation-induced central PGE_2 levels and mechanical hyperalgesia,[30] These observations are suggestive of a central site of NSAID analgesia for ketorolac which is more sensitive to the effects of NSAIDs[26] and mediates central hypersensitivity[27,30]following peripheral tissue injury. Our results are consistent with these reports, as the 30 mg ketorolac dose is sufficient to both produce analgesia and act peripherally to reduce PGE_2 levels. The 1 mg dose, however, is too low to distribute and act peripherally but sufficient to penetrate the CNS and produce analgesia due to the much greater sensitivity of the CNS to NSAIDs.

The temporal profile of TxB_2 levels in this model of tissue injury is consistent with a constitutive process that is not altered by tissue injury, suggestive of a COX–1 mediated process.

TxB_2 levels were stable, within the limits of variability, across time from the end of surgery, in the samples leading up to the onset of pain, and for the first 60 min following placebo. The concentrations of TxB_2 were rapidly suppressed by ketorolac when administered at pain onset, consistent with its dual effects on both COX-1 and COX-2. In a previous study[25] we have shown that TxB_2 gradually decreased over 4 hours in both placebo treated patients but were unaffected by the selective COX-2 inhibitor celecoxib, also supportive of TxB_2 as a biomarker for COX-1 activity.

As expected, ketorolac decreased pain dramatically following pain onset, but surprisingly there was no difference in pain relief according to route of administration. Analgesic onset was significant by 15 min following 30 mg IM ketorolac, yet changes in local PGE_2 levels were not detectable until later time points. Submucosal administration of 1 mg ketorolac resulted in a delayed onset of analgesic effect significantly greater than placebo without any effect on levels of i.r.PGE_2 at the extraction site. Intramuscular administration of 1 mg ketorolac as a control for possible absorption of drug from the peripheral site of administration also produced significant analgesia but without any change in i.r.PGE_2 at the site of injury. These observations are supportive of NSAID analgesia at some other site, presumably in the CNS, or through actions other than peripheral prostanoid suppression such as NSAID effects on acid-sensing ion channels and free radical scavenging.[31.32]

These two studies explored dynamic changes in inflammatory mediators following pain onset. These data and the results of a similar studies[12-14,24] demonstrate the utility of *in vivo* microdialysis in the oral surgery model of acute pain to study the relationships between cytokine release at the site of tissue injury, the development of acute pain, and the mechanisms of analgesic drugs.

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Table 1. Summary of demographic and surgical variables for both studies.

Study 1	N	Age	Gender M/F	Surgical Difficulty[1,2]	Lidocaine Dose (mg)	Midazolam Dose (mg)
Ketorolac 30 mg IM	30	19.7 ± 3.8	13/17	3.3 ± 0.7	86.3 ± 14.9	3.9 ± 0.9
Ketorolac Surg. Site	34	21.0 ± 4.6	20/14	3.5 ± 0.6	92.2 ± 15.7	3.9 ± 1.0
Ketorolac 1 mg IM	34	20.9 ± 4.1	18/16	3.5 ± 0.7	7.9 ±	6.0
Placebo	27	22.4 ± 5.8	18/9	3.6 ± 0.6	94.5 ± 21.7	3.9 ± 1.0
Study 2						
Ketorolac 30 mg IV	29	19.1 ± 2.9	12/17	7.1 ± 1.1	148.0 ± 16.0	4.5 ± 0.7
Placebo	28	19.6 ± 3.6	9/19	7.2 ± 1.1	146.7 ± 9.6	4.3 ± 0.7

[1] Sum of extractions classified as: 1 = simple 2 = soft tissue 3 = partial bony 4 = full bony
[2] One mandibular molar was extracted in the first study; two were extracted in the second study

Figure Legends

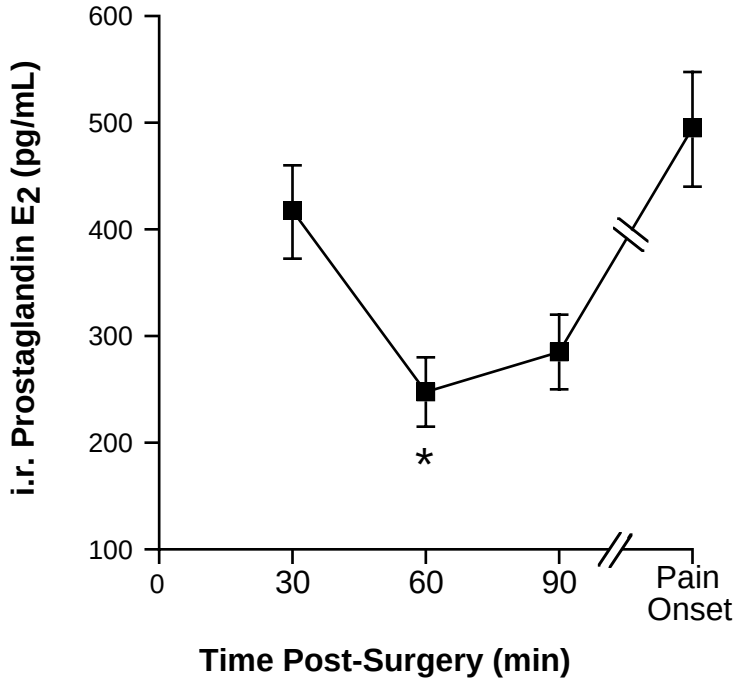
Figure 1. Postoperative immunoreactive PGE_2 concentrations at the surgical site over time: 30, 60 and 90 minutes post-surgery and at pain onset. PGE_2 concentration decreased significantly from the first postoperative sample (30 min) and then increased significantly by pain onset, prior to drug delivery. Error bars refer to SEM.

Figure 2. A. Pain intensity difference scores post-drug for single dose of ketorolac 30 mg IM, *solid triangles;* ketorolac 1 mg IM, *open diamonds*; ketorolac 1 mg submucosal, *closed squares*; and placebo, *open circles*. B. The sum of the pain intensity scores for ketorolac 30 mg IM, *diagonally striped bar*, is significantly better than all other groups; ketorolac 1 mg submucosal, *closed bar*, and ketorolac 1 mg IM, *gray bar*, are significantly better than placebo, *open bar*. Error bars refer to SEM.

Figure 3. Comparison of i.r. PGE_2 concentration at the surgical site in subjects receiving either 30 mg ketorlac IM (panel A), 1 mg ketorolac IM (panel B), or 1 mg ketorolac submucosal (panel C). Open circles represent placebo. Error bars refer to SEM.

Figure 4. Comparison of pain (panel A), i.r. prostaglandin E_2, (panel B) and i.r. thromboxane B_2 (panel C) collected by microdialysis at the surgical site. The initial observations and samples were collected over the first 60 minutes postoperatively; the remaining samples were collected at pain onset (time 0) and for 180 minutes following drug administration.

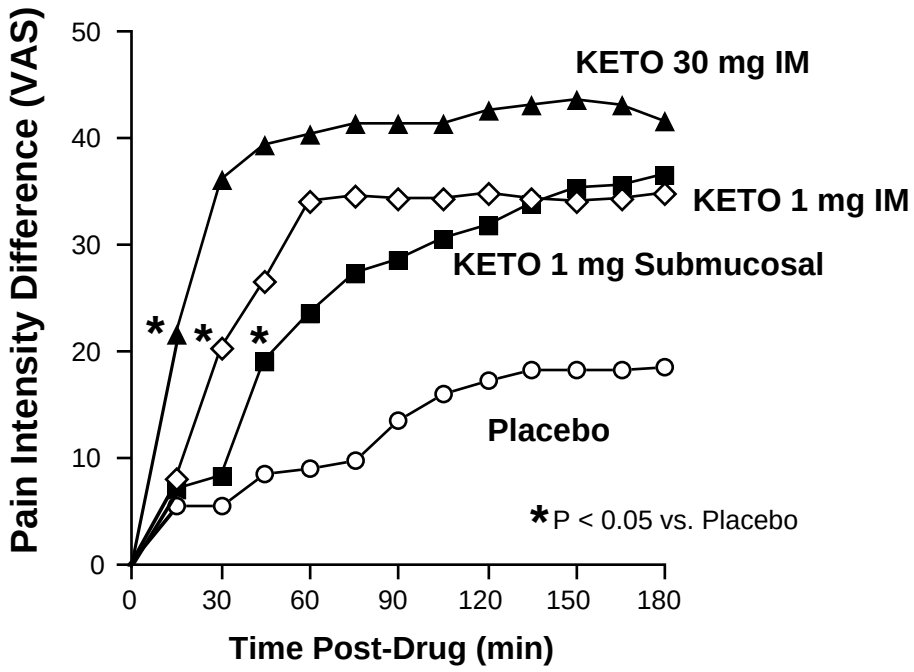
Figure 1



* P < 0.01 vs. 30 min and pain onset sample

Figure 2

A.


Pain Intensity Difference (VAS)
50
40
30
20
10
0
0
30
60
90
120
150
180
Time Post-Drug (min)
KETO 30 mg IM
KETO 1 mg IM
KETO 1 mg Submucosal
Placebo
* P < 0.05 vs. Placebo

B.

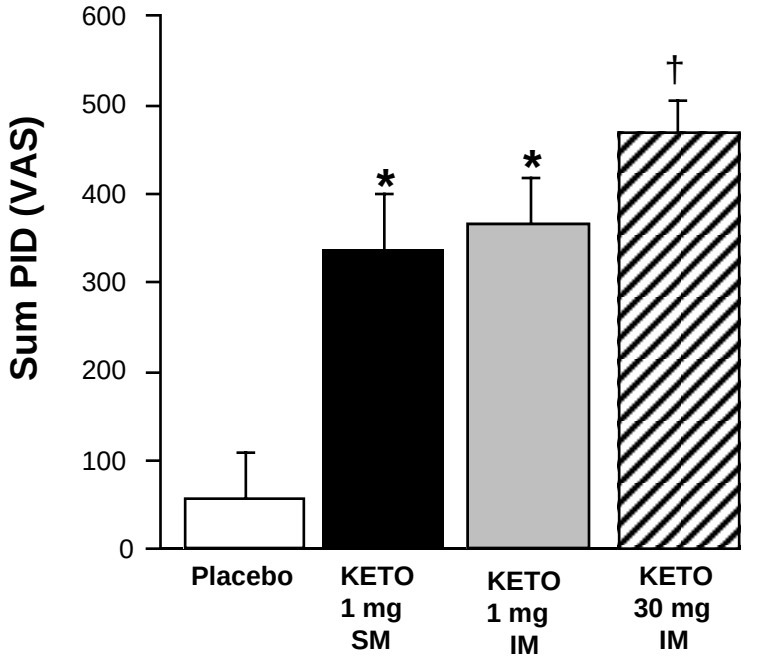
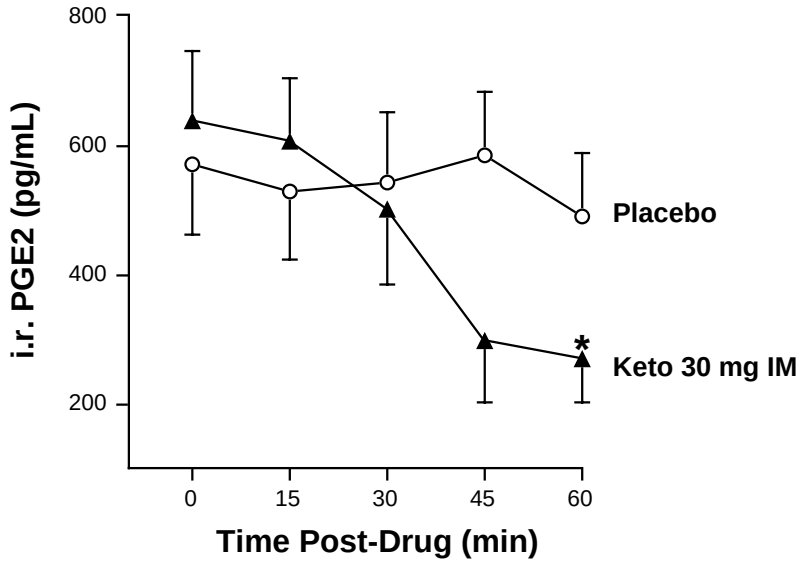

Sum PID (VAS)
600
500
400
300
200
100
0
Placebo
KETO 1 mg SM
KETO 1 mg IM
KETO 30 mg IM
* P < 0.01 vs. Placebo
† P < 0.01 vs. all groups

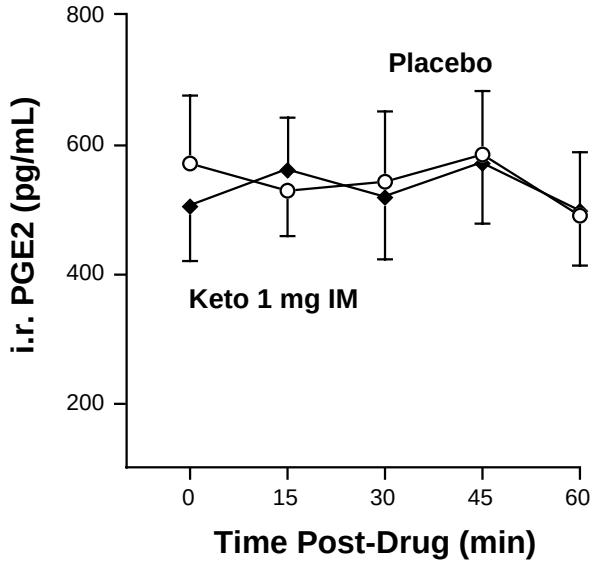
Figure 3

A.



* P < 0.05 vs. placebo

B.



C.

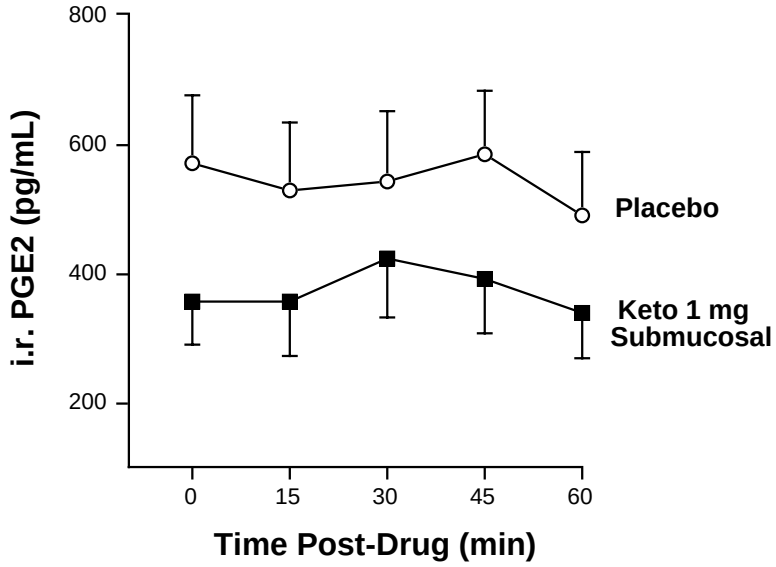
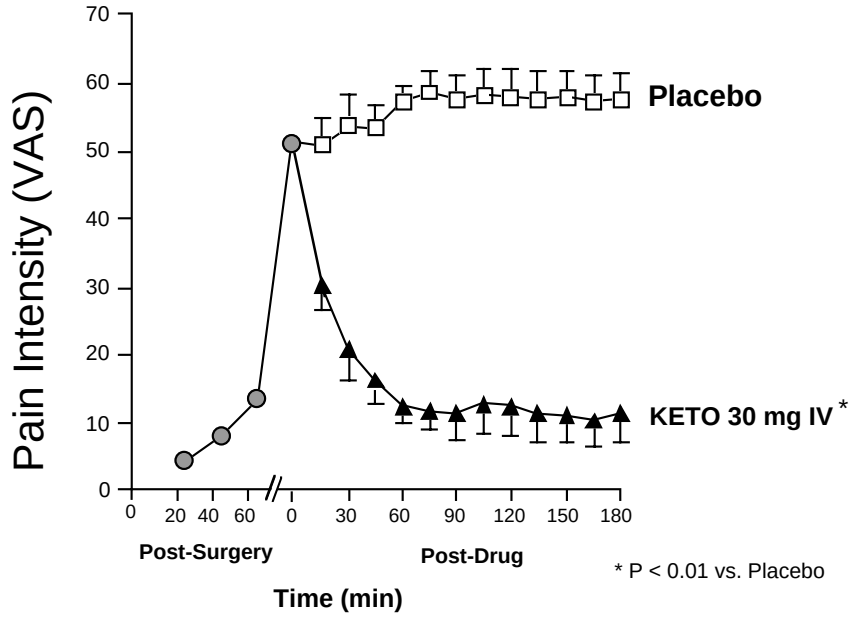
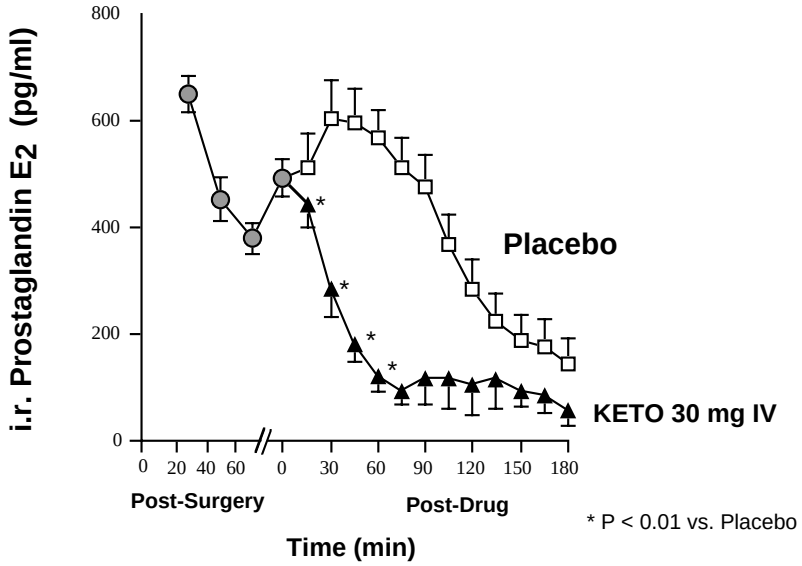


Figure 4

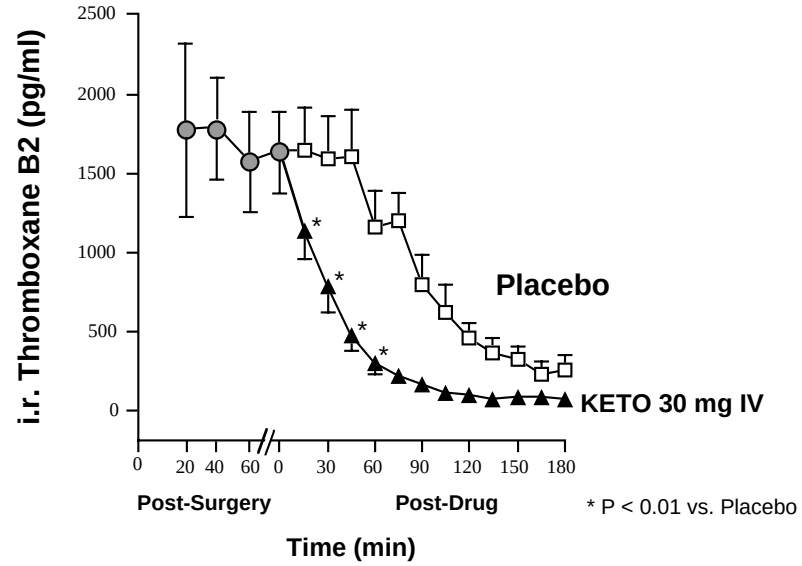
A.


Pain Intensity (VAS)
70
60
50
40
30
20
10
0
Placebo
KETO 30 mg IV *
0 20 40 60 0 30 60 90 120 150 180
Post-Surgery
Post-Drug
Time (min)
* P < 0.01 vs. Placebo

B.


i.r. Prostaglandin E2 (pg/ml)
800
600
400
200
0
Placebo
KETO 30 mg IV
0 20 40 60 0 30 60 90 120 150 180
Post-Surgery
Post-Drug
Time (min)
* P < 0.01 vs. Placebo

C.


i.r. Thromboxane B2 (pg/ml)
2500
2000
1500
1000
500
0
Placebo
KETO 30 mg IV
0 20 40 60 0 30 60 90 120 150 180
Post-Surgery
Post-Drug
Time (min)
* P < 0.01 vs. Placebo